EXHIBIT 6

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Selective Multiplication of Dihydrofolate Reductase Genes in Methotrexate-resistant Variants of Cultured Murine Cells*

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The rate of dihydrofolate reductase synthesis in the AT-3000 line of methotrexate-resistant murine Sarcoma 180 cells is approximately 200- to 250-fold greater than that of the sensitive, parental line. We have purified cDNA sequences complementary to dihydrofolate reductase mRNA and subsequently used this probe to quantitate dihydrofolate reductase mRNA and gene copies in each of these lines. Analysis of the association kinetics of the purified cDNA with DNA from sensitive and resistant cells indicated that the dihydrofolate reductase gene is selectively multiplied approximately 200-fold in the resistant line. A similar analysis of a partially revertant line of resistant cells indicated that the loss of resistance observed when the AT-3000 line is grown in the absence of methotrexate is associated with a corresponding decrease in the dihydrofolate reductase gene copy number. In each of these lines the relative number of dihydrofolate reductase gene copies is proportional to the cellular level of dihydrofolate reductase and dihydrofolate reductase mRNA sequences.

We have also studied parental and methotrexate-resistant lines of L1210 murine lymphoma cells. Both resistance and an associated 35-fold increase in the level of dihydrofolate reductase appear to be stable properties of the resistant L1210 line since we find no decrease in either parameter in over 100 generations of growth in the absence of methotrexate. Once again, we find that the increased levels of dihydrofolate reductase in the methotrexate-resistant L1210 line are associated with a proportional increase in the number of dihydrofolate reductase gene copies. In this case the dihydrofolate reductase gene copy number appears to be relatively stable in the resistant line. Therefore, we conclude that selective multiplication of the dihydrofolate reductase gene can account for the overproduction of dihydrofolate reductase in both stable and unstable lines of methotrexate-resistant cells.

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The resistance of both human neoplasms (1) and various lines of cultured cells (2-11) to the 4-amino analogs of folic acid is often associated with an increase in the cellular content of dihydrofolate reductase. We have been studying the overproduction of this enzyme in variant lines of murine Sarcoma 180 cells that were selected by a step-wise procedure for growth in the presence of high concentrations of methotrexate (a folic acid analogue). Dihydrofolate reductase comprises as much as 6% of the soluble protein in the methotrexate-resistant AT-3000 line, representing an increase of more than 200-fold over the level in the sensitive, parental cells (12). Purified dihydrofolate reductase from resistant cells appears to be identical to that from sensitive cells; and, in addition, the relative half life of the enzyme is similar to these lines (12). We have demonstrated that the increased level of dihydrofolate reductase in resistant cells is due to an increased rate of enzyme synthesis (12), and that, in turn, this increase is correlated with increased cellular levels of translatable dihydrofolate reductase mRNA (13).

One of the most interesting characteristics of the AT-3000 line is that high levels of resistance are lost when these cells are grown in the absence of methotrexate (3). Loss of resistance is associated with a decrease in the level of dihydrofolate reductase (3, 12), and a corresponding decrease in both the rate of dihydrofolate reductase synthesis (12) and the level of the specific mRNA activity (13). Several lines of evidence suggest that these decreases are due to the instability of the variation (mutation?) which leads to increased enzyme synthesis (12). Instability is also a characteristic of methotrexate resistance in a number of other cell lines (9, 14). In contrast, in certain lines of methotrexate-resistant baby hamster kidney (BHK) cells (15) as well as in other resistant lines (2), resistance and increased levels of dihydrofolate reductase appear to be stable characteristics and do not decline when the cells are grown in the absence of the drug. However, other properties of methotrexate resistance in BHK cells appear to be similar to those of Sarcoma 180 cells, including high rates of dihydrofolate reductase synthesis (16) and high levels of translatable dihydrofolate reductase mRNA (17). Various considerations of the possible mechanisms that could lead to stable or unstable changes in the phenotypic expression

of cultured cells have been discussed (18, 19).

We report here the purification of cDNA sequences complementary to dihydrofolate reductase mRNA of murine origin, and subsequent use of this probe to quantitate dihydrofolate reductase mRNA and gene copies in a number of different cell lines. We have examined both sensitive and methotrexate-resistant lines of Sarcoma 180 cells, as well as a partially revertant line that was derived by growing resistant cells in the absence of methotrexate for 400 cell doublings (12). In addition, we have also studied parental and methotrexate-resistant lines of L1210 murine lymphoma cells. Both resistance and associated high levels of dihydrofolate reductase appear to be stable properties of the L1210 lines since we find no decrease in either parameter over several hundred generations of growth in the absence of methotrexate. We find that in both the stable (L1210) and unstable (S-180) lines of resistant cells, increased levels of dihydrofolate reductase and dihydrofolate reductase mRNA are associated with a proportional increase in the number of dihydrofolate reductase gene copies. When unstable lines are grown in the absence of selection, loss of resistance is associated with a decrease in the dihydrofolate reductase gene copy number.

EXPERIMENTAL PROCEDURES

Materials—Sources of most of the reagents have been given previously (12, 13). Oligo(dT)-cellulose and oligo(dT)$_{12-18}$ were purchased from Collaborative Research; micrococcal nuclease, salmon sperm DNA, and calf thymus DNA from Sigma; S1 nuclease from Miles; [^{3}H]leucine (5 Ci/mmol) from New England Nuclear; [^{3}H]deoxycytidine triphosphate (20 Ci/mmol) from Amersham/Searle; Chelex 100 and Bio-Gel hydroxylapatite from Bio-Rad. Purified reverse transcriptase (Lot no. G-1176, 39,216 units/mg) was supplied by Dr. J. W. Beard (Life Sciences Inc., St. Petersburg, Florida) and methotrexate by Dr. Paul Davignon, Pharmaceutical Resources Branch, National Cancer Institute.

Generously provided as gifts were purified ovalbumin mRNA and *E. coli* tRNA from Dr. Gray Crouse (Stanford University), and purified chicken oviduct DNA from Dr. Henry Burr (Stanford University).

Cell Culture—The Sarcoma 180 cell line and the 3000-fold methotrexate-resistant AT-3000 subline were grown as described previously (12) except that thymidine and glycine were omitted from the medium of the resistant cells. A partially phenotypic revertant line, Rev-400, was obtained by growing the AT-3000 line for 400 cell doublings in methotrexate-free medium. Some characteristics of the "revision" phenomenon have been described previously (12), and further details are described under "Results."

Suspensions of L1210 murine lymphoma cells (L1210S) were grown in Fischer's Medium for Leukemic Cells of Mice (GIBCO) containing 10% horse serum. A 5000-fold methothrexate-resistant subline (L1210RR) and a 25,000-fold resistant subline (L1210 RR500) were grown in the same medium supplemented with 100 μM and 500 μM methotrexate, respectively. For some of the experiments described the L1210RR line was grown for approximately 100 cell doublings (over 10 months) in methotrexate-free medium. Further characteristics of these lines will be described under "Results" and elsewhere.[1]

Determination of the Relative Rate of Dihydrofolate Reductase Synthesis—The relative rate of dihydrofolate reductase synthesis was determined as described previously by direct immunoprecipitation of the enzyme from extracts of pulse-labeled cells (12).

RNA Preparation—Total cytoplasmic RNA was prepared from each of the cell lines as described previously (13). These preparations were used immediately or stored in liquid nitrogen.

Poly(A)-containing RNA was prepared by oligo(dT)-cellulose chromatography of total cytoplasmic RNA. RNA was dissolved in 10 mM Tris/Cl (pH 7.4) and 0.5% sodium dodecyl sulfate, heated at 68° for 5 min and rapidly cooled in an ethanol-ice bath. This solution was then adjusted to 0.4 M NaCl and oligo(dT)-cellulose chromatography was carried out essentially as described by Aviv and Leder (20). The bound RNA fraction was eluted with 10 mM Tris/Cl (pH 7.4) and 0.5% sodium dodecyl sulfate, adjusted to 400 mM NaCl, and precipitated overnight at −20° by the addition of 2 volumes of ethanol. The precipitates were dissolved in a minimal volume of H_2O and stored in liquid N_2.

Polysome Preparation—The various lines were grown in roller bottles and were fed with fresh medium 4 h prior to harvest. Cells were rinsed once with ice cold Hanks' balanced salts solution plus 50 μg/ml cycloheximide, scraped from the bottles with rubber policemen, and washed three times by centrifugation through the same salt solution. Homogenization (13) and preparation of polysomes by the "cushion" method was as described previously by Palacios *et al.* (21), except that the homogenization buffer contained 10 mM $MgCl_2$. Polysomes were dialyzed for 12 h against 25 mM Tris/Cl (pH 7.1), 25 mM NaCl, 5 mM $MgCl_2$, and 1 mg/ml sodium heparin (Buffer A) and then stored in liquid nitrogen for subsequent use.

Antibody Purification—Rabbit anti-dihydrofolate reductase γ-globulin, prepared against purified dihydrofolate reductase protein as described previously (12), was purified by affinity chromatography on dihydrofolate reductase-Sepharose. Conditions for preparation of the resin and affinity chromatography were essentially as described by Shapiro *et al.* (22). Bound γ-globulin, eluted with 4.5 M $MgCl_2$, was enriched approximately 100-fold for anti-dihydrofolate reductase activity. The purified antibody preparation was made ribonuclease-free by passage through a column of DEAE-cellulose overlaid with CM-cellulose (21).

Iodination of Anti-dihydrofolate Reductase Globulin—Anti-dihydrofolate reductase globulin was iodinated by the lactoperoxidase method essentially as described by Taylor and Schimke (23). Iodinated antibody was made ribonuclease-free as described above.

Binding of Iodinated Anti-dihydrofolate Reductase Antibody to Polysomes—Prior to incubation, polysomes prepared as described above were thawed at 4° and centrifuged for 10 min at 5000 × *g* to remove particulate material. Reaction mixtures containing 30 A_{260} units of polysomes and 1.3 μg of iodinated anti-dihydrofolate reductase (specific radioactivity 77,000 cpm/μg) in 2 ml of Buffer A were incubated for 50 min at 0°. Polysomes were then reisolated from the reaction mixture by the "cushion" method as described above and sedimented through a linear sucrose gradient (0.6 M to 1.5 M in 11 ml of Buffer A) for 1.8 h at 4°. Gradients were fractionated and monitored for A_{260} with an Isco model 640 density gradient fractionator equipped with an ultraviolet flow monitor. For scintillation counting, 0.5-ml fractions were dissolved in 10 ml of Instagel (Packard).

Isolation of Dihydrofolate Reductase-synthesizing Polysomes—Indirect immunoprecipitation of polysomes was carried out essentially as described by Shapiro *et al.* (22). Resistant cell (AT-3000) polysomes at a final concentration of 10 to 15 A_{260} units/ml in 25 mM Tris/Cl (pH 7.1), 4 mM $MgCl_2$, 150 mM $NaCl_4$, 750 μg/ml sodium heparin, and 0.5% w/v Triton X-100 and sodium deoxycholate were incubated with optimal concentrations of purified rabbit anti-dihydrofolate reductase γ-globulin (20 μg/A_{260} unit of polysomes) for 60 min at 0°. The antibody·nascent chain complex was then precipitated by incubation with goat anti-rabbit γ-globulin (80 μg/μg of rabbit γ-globulin) for an additional 90 min at 0°. The precipitated complex was pelleted and washed as described by Shapiro *et al.* (22). Pellets were resuspended in 25 mM Tris/Cl (pH 7.1), 5 mM EDTA, 6 mM $MgCl_2$, 25 mM NaCl, 1 mg/ml sodium heparin, and 1% sodium dodecyl sulfate, and RNA was extracted by the phenol/chloroform procedure described previously (13).

RNA-dependent Rabbit Reticulocyte Lysates—Micrococcal nuclease-treated rabbit reticulocyte lysates were prepared by a modification of the procedure described by Pelham and Jackson (24) as follows: standard rabbit reticulocyte lysate reaction mixtures were prepared as described previously (13) except that [^{3}H]leucine and RNA were omitted. Aliquots (325 μl) of this mixture were combined with 3.3 μl of 100 mM $CaCl_2$ (final concentration, 1 mM) and 3.3 μl of a 1 mg/ml solution of micrococcal nuclease (final concentration 10 μg/ml), and incubated for 15 min at 25°, at which time nuclease action was inhibited by the addition of 7 μl of 100 mM ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid (final concentration, 2 mM). The nuclease-treated reticulocyte lysate mix prepared in this fashion was either used immediately or stored for up to 2 weeks in liquid nitrogen with no significant loss of activity.

Typical *in vitro* protein synthesis assays consisted of 60 μl of nuclease-treated lysate reaction mix, 4.6 μl of 200 μM [^{3}H]leucine

[1] C. Lindquist and J. Bertino, manuscript in preparation.

(specific radioactivity, 5 Ci/mmol), and 25.4 μl of an aqueous solution of RNA. Following incubation for 1 h at 25°, the reaction was terminated by the addition of 36 μl of 0.1 M leucine and 14 μl of a mixture of 10% (w/v) sodium deoxycholate and 10% (w/v) Triton X-100. Stimulation of total protein synthesis was determined as the difference between trichloroacetic acid-precipitable radioactivity appearing in reactions that had received RNA and that in reactions to which no RNA had been added. Incorporation into dihydrofolate reductase was measured by specific immunoprecipitation as described previously (13) and expressed as a percentage of the total stimulated trichloroacetic acid-precipitable radioactivity in the lysate reaction. Under these conditions, incorporation into total trichloroacetic acid-precipitable radioactivity and dihydrofolate reductase was linear with time for up to 90 min with added poly(A)-containing RNA to 15 μg/ml, and with added total RNA to at least 50 μg/ml. Typical stimulation for a standard translation assay was approximately 100,000 cpm/μg of poly(A)-containing RNA, a level 20- to 30-fold greater than background.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Lysate Products – After termination of lysate reactions, aliquots were removed and mixed with an equal volume of dissolving buffer, boiled for 3 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (12). Subsequent to electrophoresis, gels were soaked for 16 h in a liter of 7.5% acetic acid and 5% methanol (with one change of solution) in order to remove soluble radioactivity. Gels were then sliced and prepared for scintillation counting as described previously (12).

cDNA Preparation – cDNA was prepared essentially as described by Buell *et al.* (25). The reactions were carried out in 20-μl volumes and contained: 50 mM Tris/Cl (pH 8.3), 140 mM KCl, 30 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 μg/ml oligo(dT), 0.5 mM dGTP, dATP, and dTTP, 0.5 mM [^{3}H]dCTP (20 Ci/mmol), 16 units of avian myeloblastosis virus reverse transcriptase, and 3 μg of poly(A)-containing RNA prepared from either total cytoplasmic-resistant cell RNA, or RNA extracted from immunoprecipitated dihydrofolate reductase synthesizing resistant cell polysomes.

Reactions were incubated at 42° for 1 h and stopped by the addition of 120 μl of 0.3 M NaOH. After a further incubation at 37° for 20 h, samples were neutralized with 1 N HCl and sodium dodecyl sulfate was added to a final concentration of 0.1%. The reaction mixtures were then extracted with 2 volumes of $CHCl_3$, and the aqueous phase passed over a small (8-ml) column of G-100 Sephadex which was previously equilibrated with H_2O. The void volume was pooled and concentrated by ethanol precipitation.

In all cases the yield was approximately 10^6 cpm of cDNA per μg of added RNA. Based on the specific radioactivity of the [^{3}H]dCTP and assuming equal representation of all four bases, this corresponds to approximately 0.1 μg of cDNA synthesized per μg of added RNA. Approximately 7 to 8% of the trichloroacetic acid-precipitable radioactivity in the cDNA preparation was resistant to treatment with S1 nuclease.

RNA/cDNA Hybridizations – All analytical RNA/cDNA hybridizations were done in 20 mM Tris/Cl (pH 7.7), 600 mM NaCl, 2 mM EDTA, and 0.2% sodium dodecyl sulfate except where noted otherwise. Reaction mixtures of 2 to 40 μl were overlaid with mineral oil in plastic tubes and incubated at 68°. The quantities of [^{3}H]cDNA and RNA used in these reactions are described in appropriate figure legends. In all cases, final R_0t values were corrected to standard salt conditions (26).

At the end of the incubation, reaction mixtures were diluted into 1 ml of buffer containing 30 mM Na ($C_2H_3O_2$) (pH 4.5), 3 mM $ZnSO_4$, 300 mM NaCl, and 10 μg/ml denatured salmon sperm DNA. Each sample was divided into two aliquots: one was digested for 30 min at 45° with 8 μg/ml of S1 nuclease, and the other incubated identically, but without S1 nuclease. After digestion, 100 μg/ml of carrier calf thymus DNA was added to both S1-treated and control samples and nucleic acids precipitated with an equal volume of 10% trichloroacetic acid containing 1% sodium pyrophosphate at 4° for 15 min. Precipitates were collected on Millipore filters, washed three times with 5% trichloroacetic acid, dried, and counted in 10 ml of ScintiLene (Fisher).

Hybrid formation was scored as the amount of trichloroacetic acid-precipitable radioactivity remaining after S1 treatment and expressed as a percentage of the untreated control value. Depending on the cDNA preparation from 1.5 to 8% of the trichloroacetic acid-precipitable counts were resistant to S1 treatment in the absence of added RNA. In all experiments, the appropriate percentage of endogenous S1 resistance was subtracted from treated and control values before calculation of the per cent hybridization. In calculating R_0t values, we assumed an average value of 346 g of RNA nucleotides per mol.

DNA Preparation – The 27,000 × *g* pellets (containing nuclei) resulting from standard RNA preparations (13) were stored at −20°. Approximately 5 ml of frozen nuclear pellet was thawed and gently homogenized in 50 ml of 0.15 M NaCl, 0.1 M EDTA (pH 8.0), 0.6 M sodium perchlorate, and 1.0% sodium dodecyl sulfate by five strokes in a dounce homogenizer (loose pestle). The homogenate was slowly stirred at 25° for 30 min, extracted with 2 volumes of chloroform, and DNA was spooled from the aqueous phase after the addition of 2 volumes of ice cold ethanol.

Spooled DNA was dissolved in 10 mM Tris/Cl (pH 7.4) and then treated with 50 μg/ml pancreatic ribonuclease (boiled for 10 min in 20 mM NaCl prior to use) for 2 h at 37°. Sodium dodecyl sulfate and proteinase K were then added to a final concentration of 0.2% and 50 μg/ml, respectively, and the incubation continued for another 5 h at 37°. The solution was then extracted with 2 volumes of $CHCl_3$ and the aqueous phase precipitated overnight at −20° with 2 volumes of ethanol. Precipitated DNA was pelleted by centrifugation for 5 min at 2000 × *g*, lyophilized, and dissolved in 100 mM sodium acetate (pH 7.8). DNA was then sheared by passage through the needle valve of a French pressure cell at a pressure of 20,000 p.s.i. Divalent cations were removed by passing the sheared DNA preparations over a small (10 ml) volume of Chelex (equilibrated with 100 mM sodium acetate, pH 7.8), and the DNA was subsequently ethanol-precipitated as described above and redissolved in 20 mM Tris/Cl (pH 7.4) and 1 mM EDTA. 1 M NaOH was then added to a final concentration of 0.3 M and the solution was incubated for 22 h at 37°, at which time the base was neutralized by the addition of an equivalent amount of 1 N HCl.

These preparations were then stored at 4° until subsequent use as described below. All of the DNA samples prepared in this fashion sedimented as symmetrical peaks on isokinetic alkaline sucrose gradients (see below) with a calculated size of approximately 450 base pairs.

Sedimentation Analysis of DNA – DNA was analyzed by sedimentation through isokinetic alkaline sucrose gradients prepared as described by McCarty *et al.* (27) using 5% and 29.4% sucrose containing 0.1 N NaOH and 0.9 M NaCl. The molecular size of the DNA was calculated from S value as described by Studier (28).

cDNA/DNA Association Reactions – DNA/DNA associations were done in reaction mixtures containing 25 mM Tris/Cl (pH 7.4), 1 mM EDTA, 300 mM NaCl, 50 pg of [^{3}H]cDNA (500 cpm), and 500 μg of cellular DNA (prepared as described above) in a final volume of from 0.05 ml to 1.1 ml. Reaction mixtures were overlaid with mineral oil in plastic tubes, heated to 102° for 10 min in an H_2O/ethylene glycol bath, cooled, and incubated at 68° for various times in order to achieve the desired C_0t values.

Single- and double-stranded DNA were then fractionated by chromatography on hydroxylapatite. Reaction mixtures were diluted into 5 ml of 0.12 M $NaPO_4$ (pH 6.8) and passed over a column containing 1 g of hydroxylapatite (boiled for 5 min in 5 ml of 0.12 M $NaPO_4$ prior to use and equilibrated in the same buffer) which was maintained at 60° with a recirculating water bath. Single-stranded DNA was eluted with 0.12 M $NaPO_4$ (pH 6.8) and double-stranded material subsequently eluted with 0.5 M $NaPO_4$ (pH 6.8). The single- and double-stranded fractions were monitored for A_{260}, and the DNA then was precipitated by the addition of carrier calf thymus DNA to 25 μg/ml and 0.1 vol of 100% trichloroacetic acid. Trichloroacetic acid-precipitated material was collected and counted as described above. In order to calculate DNA concentration, an A_{260} absorbance of 1 was assumed to correspond to DNA concentrations of 43 μg/ml and 50 μg/ml, respectively, for single- and double-stranded DNA fractions. The per cent double-stranded in each sample was determined by dividing the amount of DNA or [^{3}H]cDNA recovered in the double-stranded fraction by the total amount recovered in the double- and single-stranded fractions. In calculating C_0t values we assumed an average value of 332 g of DNA nucleotides per mol.

RESULTS

Purification of Dihydrofolate Reductase-specific cDNA

Immunoprecipitation of Dihydrofolate Reductase-synthesizing Polysomes – In order to further study the factors responsible for the accumulation of high levels of translatable dihydro-

folate reductase mRNA in methotrexate-resistant cells, we needed a cDNA probe complementary to dihydrofolate reductase mRNA. The usual method for the preparation of such a reagent has involved purification of a specific mRNA and subsequent synthesis of a complementary cDNA. Dihydrofolate reductase mRNA contains poly(A), allowing easy separation from rRNA, but its sedimentation rate on sodium dodecyl sulfate or denaturing sucrose gradients is not sufficiently distinct from that of total poly(A)-containing RNA to permit a significant additional purification by size fractionation (13). Therefore, we have employed the specific polysome immunoprecipitation procedure described by Shapiro *et al.* (22) to enrich for dihydrofolate reductase-synthesizing polysomes. The initial step in this procedure involved incubation of purified (100-fold) anti-dihydrofolate reductase antibody with resistant cell polysomes. The data in Fig. 1*a* demonstrate that this procedure results in the specific binding of the antibody to a size class of resistant cell polysomes (5 to 7 ribosomes) expected for those engaged in the synthesis of dihydrofolate reductase (M_r = 20,000). However, only a low level of apparently nonspecific binding is observed with polysomes from sensitive cells (Fig. 1*b*), where the rate of dihydrofolate reductase synthesis is below the resolution level of this technique. These results suggest that the incubation procedure results in the binding of purified antibody specifically to dihydrofolate reductase nascent chains. Subsequent to the initial binding reaction, the resulting antibody·nascent chain·polysome complexes were precipitated with a second antibody directed against the first antibody (see "Experimental Procedures" for details). We estimated the purification achieved by this procedure by translating the poly(A)-containing RNA extracted from the immunoprecipitated polysomes in the mRNA-dependent rabbit reticulocyte lysate (25). At the end of the incubation, samples of the total lysate reaction mix were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Fig. 2*a* indicates the very low background observed in this system in the absence of added RNA. In contrast, addition of purified ovalbumin mRNA resulted in the stimulation of a single peak of incorporated radioactivity with a mobility characteristic of authentic ovalbumin (Fig. 2*b*). This result indicates that the generation of incomplete or fragmented polypeptide chains is not a problem with this system. Furthermore, the specificity of the assay is evidenced by the fact that in this experiment more than 95% of the stimulated incorporation was precipitable with anti-ovalbumin antibody (data not shown). The addition of polysomal poly(A)-containing RNA from resistant cells resulted in the synthesis of a broad size distribution of proteins of which approximately 1.9% were precipitable by anti-dihydrofolate reductase antibody (data not shown). This value corresponds well to our estimate of the relative rate of dihydrofolate reductase synthesis as a per cent of total protein synthesis in this line.[2] Poly(A)-containing RNA extracted from the immunoprecipitated polysomes stimulated incorporation into a single major peak of radioactivity which co-migrated with added dihydrofolate reductase marker (Fig. 2*d*). In this experiment, 25% of the stimulated incorporation was precipitable by anti-dihydrofolate reductase antibody (data not shown). Comparison of the relative incorporation into dihydrofolate reductase

[2] We have previously described dihydrofolate reductase synthesis as a per cent of soluble protein synthesis. In these lines, soluble protein accounts for approximately 20 to 30% of the total protein synthesis (data not shown).

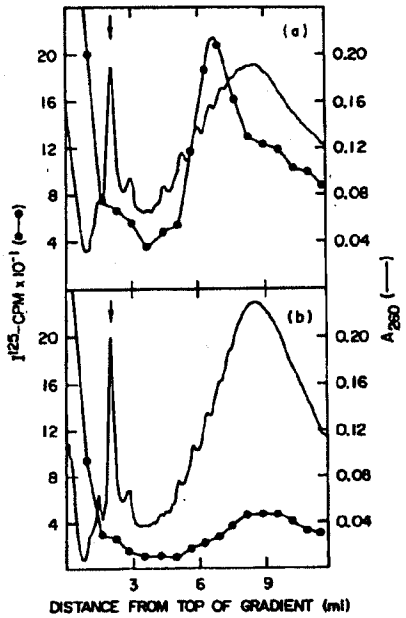

FIG. 1. Binding of anti-dihydrofolate reductase antibody to polysomes. The binding of ^{125}I-labeled anti-dihydrofolate reductase antibody to polysomes from AT-3000 (*a*) and S-3 (*b*) cells was examined as described under "Experimental Procedures." ^{125}I-radioactivity, ●—●; A_{260}, ——.

in the experiments presented in Fig. 2, *c* and *d* indicates an approximately 10-fold purification of dihydrofolate reductase mRNA by the polysome precipitation procedure.

cDNA Synthesis from the Partially Purified Dihydrofolate Reductase mRNA – cDNA was prepared from the partially purified dihydrofolate reductase mRNA resulting from the immunoprecipitation procedure and then analyzed by hybridization to excess poly(A)-containing RNA from sensitive or resistant cells (Fig. 3*a*). Comparison of the kinetics of these reactions indicates that approximately 15 to 20% of the cDNA sequences hybridize to mRNA sequences that are considerably more abundant in resistant cells than in sensitive cells. This percentage roughly corresponds with our estimate of the proportion of dihydrofolate reductase mRNA sequences in the partially purified RNA preparation from which the cDNA was synthesized (see above). However, these data indicate that this cDNA preparation is not pure enough for use as an analytical reagent. In order to further enrich for cDNA sequences complementary to the dihydrofolate reductase mRNA, we devised the purification scheme described below.

Purification of cDNA Sequences Complementary to Dihydrofolate Reductase mRNA – As a further means of purification of dihydrofolate reductase-specific sequences in the cDNA preparation, we exploited the large and apparently specific increase in the abundance of dihydrofolate reductase mRNA sequences in the RNA population of resistant as compared to sensitive cells. Analysis of the soluble proteins produced by sensitive and resistant cells suggested that the only major difference between the two is the overproduction of dihydrofolate reductase (12). Furthermore, we have used a reticulocyte lysate *in vitro* translation assay to demonstrate that most, if not all, of the several hundred-fold increase in the level of dihydrofolate reductase synthesis in resistant cells can be

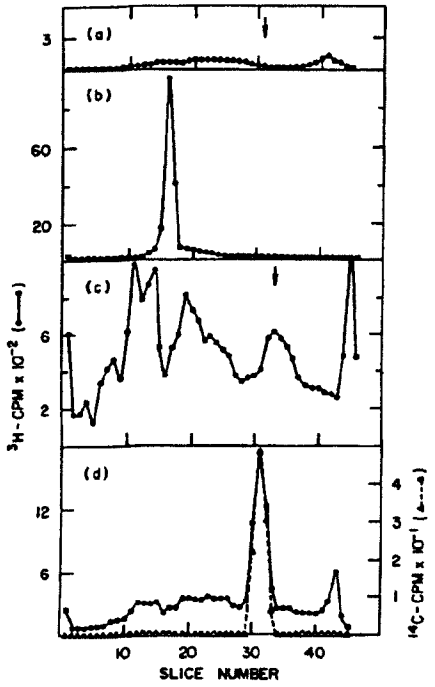

FIG. 2. Electrophoretic analysis of mRNA-dependent rabbit reticulocyte reaction products. Aliquots from mRNA-dependent lysate reactions stimulated with (*a*) no added RNA, (*b*) 1.5 μg of purified ovalbumin mRNA, (*c*) 1.25 μg of poly(A)-containing RNA prepared from AT-3000 cell polysomes, (*d*) 1 μg of poly(A)-containing RNA prepared from immunoprecipitated dihydrofolate reductase-synthesizing polysomes (see "Experimental Procedures" for details) were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures." Other aliquots of the stimulated lysate reactions were used for the specific immunoprecipitation of ovalbumin (*reaction b*) as described by Rhoads *et al.* (29) and dihydrofolate reductase (*reactions c* and *d*) as described previously (12). The incorporation into each of these proteins relative to total stimulation was measured as described under "Experimental Procedures." ^{3}H-labeled lysate reaction products, ●——●; authentic ^{14}C-labeled dihydrofolate reductase, △- - -△. *Arrows* mark the migration of added ^{14}C-labeled dihydrofolate reductase in *panels a* and *c*.

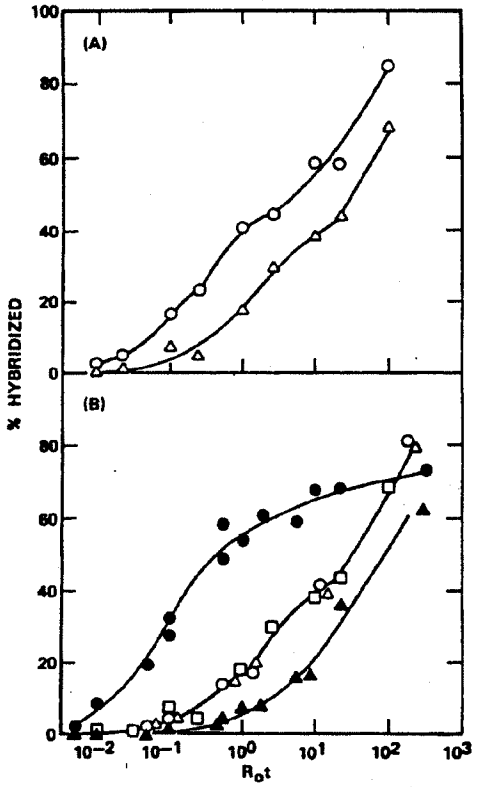

FIG. 3. *A*, hybridization of cDNA prepared from partially purified dihydrofolate reductase mRNA to poly(A)-containing RNA from resistant and sensitive S-180 cells. Poly(A)-containing RNA extracted from the S-3 (△——△) and AT-3000 (○——○) cell lines was reacted with 60 pg (600 cpm) of [^{3}H]cDNA prepared from partially purified dihydrofolate reductase mRNA (see text). Similar quantities of RNA from sensitive and resistant lines, ranging from 0.1 μg to 1 μg per sample were used to drive hybridization reactions that were stopped at corresponding R_0t values. Other reaction conditions and measurement of the extent of hybridization by S1 nuclease hydrolysis are described under "Experimental Procedures." Endogenous S1 resistance of this cDNA preparation was approximately 8%. *B*, hybridization of partially purified cDNA to RNA from sensitive and resistant cells. The [^{3}H]cDNA recovered in the single- and double-stranded fractions resulting from *Step A* of the purification procedure outlined in Fig. 4 was hybridized to excess poly(A)-containing RNA from S-3 and AT-3000 cells. Reaction conditions were essentially as described in *A*. Hybridization to AT-3000 RNA: [^{3}H]cDNA from single- (●——●) and double-stranded (○——○) fractions; hybridization to S-3 RNA: [^{3}H]cDNA from single- (▲——▲) and double-stranded (△——△) fractions. Hybridization of unfractionated [^{3}H]cDNA to S-3 RNA is reproduced from *A* (□——□).

attributed to a similar increase in the level of dihydrofolate reductase mRNA activity (13). Therefore, we estimate that dihydrofolate reductase mRNA sequences are as much as 200 to 300 times more abundant in resistant cells than in sensitive cells, whereas other mRNA sequences are probably present in similar abundance in the two cell types. Furthermore, since the cDNA was prepared from resistant cell RNA that was enriched an additional 10-fold for dihydrofolate reductase mRNA sequences, the dihydrofolate reductase-specific sequences in the cDNA preparation could be as much as 2000- to 3000-fold more abundant than the complementary sequences in sensitive cell poly(A)-containing RNA. These estimates provide the rationale for Step A of the dihydrofolate reductase-specific cDNA purification procedure that is outlined in Fig. 4. The cDNA preparation was incubated with a 30-fold mass excess of sensitive cell poly(A)-containing RNA to a R_0t value sufficiently high to ensure completion of the reaction (see legend to Fig. 4). Under these conditions, cDNA complementary to mRNA sequences that are present in similar abundance in resistant and sensitive cells (or in greater abundance in sensitive cells) should be driven into hybrids by the excess sensitive cell RNA. However, the majority of the cDNA sequences that are complementary to mRNA sequences present in far greater abundance in resistant cells than in sensitive cells (relative to the 30-fold mass excess of sensitive cell RNA) will remain single-stranded at the end of the reaction. Therefore, based on the estimates described above, these unhybridized cDNA sequences should be greatly enriched for sequences complementary to dihydrofolate reductase mRNA.

Subsequent to the hybridization reaction, single- and dou-

ble-stranded material was separated by chromatography on hydroxylapatite, RNA removed by alkaline hydrolysis, and the cDNA from both fractions analyzed by hybridization to excess RNA from sensitive and resistant cells. The cDNA recovered in the double-stranded fraction should contain sequences present at a similar abundance in both cell types. As expected, this cDNA fraction hybridized to RNA from sensitive (Fig. 3*B*, △——△) and resistant (Fig. 3*B*, ○——○) cells with kinetics that were essentially identical to each other and to those with which the unfractionated cDNA preparation hybridized to RNA from sensitive cells (Fig. 3*B*, □——□). However, most of the cDNA recovered in the single-stranded fraction hybridized to excess RNA from resistant cells (Fig. 3*B*, ●——●) at a rate approximately 200-fold greater than to that of sensitive cells (Fig. 3*B*, ▲——▲). This difference is consistent with our estimate of the relative level of dihydrofolate reductase mRNA sequences in these cell types. We recovered approximately 23% of the unfractionated cDNA in the single-stranded fraction, and of this about 65 to 70% had highly accelerated kinetics when hybridized to RNA from resistant cells as opposed to that of sensitive cells. Assuming that the relative abundance of sequences in the cDNA preparation is representative of the mRNA population from which it was derived, this recovery roughly corresponds to that expected for dihydrofolate reductase-specific sequences. The maximum hybridization observed with this cDNA fraction was never above 80%. Presumably, the explanation for this result is that in selecting for single-stranded material after the hybridization described above, we also enrich for any nonhybridizable material present in the unfractionated cDNA preparation.

Step A

1. Poly(A)-containing RNA from resistant cells (enriched for dihydrofolate reductase sequences)

↓

[^{3}H]cDNA

↓

2. Hybridized to 30-fold mass excess of poly(A)-containing RNA from sensitive cells (final R_0t = 1600 mol-s/liter)

↓

3. Nonhybridized [^{3}H]cDNA recovered by hydroxylapatite chromatography

Step B

1. [^{3}H]cDNA selected by Step A hybridized to large excess of resistant cell poly(A)-containing RNA (final R_0t = 0.8 mol-s/liter)

↓

2. Hybridized [^{3}H]cDNA recovered by hydroxylapatite chromatography

FIG. 4. Purification of cDNA sequences complementary to dihydrofolate reductase mRNA. *Step A*, hybridization to a limited excess of sensitive cell poly(A)-containing RNA. Approximately 200 ng of [^{3}H]cDNA that was prepared from resistant cell (AT-3000) poly(A)-containing RNA extracted from partially purified dihydrofolate reductase-synthesizing polysomes (see "Experimental Procedures" and text for details) was hybridized to 6 μg of sensitive cell (S-3) poly(A)-containing RNA (30-fold mass excess of RNA). The final reaction volume was 20 microliters, and the other conditions were as described under "Experimental Procedures." The extent to which the preparative reaction approached maximum hybridization was estimated at various times (R_0t values) by measuring the S1 nuclease resistance of control samples identical to the preparative reaction just described except that only 400 pg of [^{3}H]cDNA was used (15,000-fold mass excess of RNA). At an R_0t of 1600 mol-s/liter, a value where the cDNA in the control samples was essentially 100% S1 nuclease-resistant, the preparative sample was diluted with 68 μl of H_2O containing 15 μg each of native and denatured salmon sperm DNA (sheared to 400 base pairs) and 12 μl of 1 M $NaPO_4$ (final concentration, 0.12 M). Single- and double-stranded material was then fractionated by chromatography on hydroxylapatite essentially as described under "Experimental Procedures." Approximately 23% of the radioactivity failed to bind to the column in 0.12 M $NaPO_4$. This represented single-stranded material since greater than 93% was sensitive to S1 nuclease digestion. The remainder of the cDNA was eluted in the double-stranded fraction with 0.4 M $NaPO_4$ and was essentially 100% resistant to S1 nuclease. RNA was removed from the double-stranded fraction by base hydrolysis (see "Experimental Procedures" for details) and the cDNA from each of these fractions was tested by hybridization to excess poly(A)-containing RNA from resistant and sensitive cells. (See text and legend to Fig. 3*B* for details.) *Step B*, low R_0t fractionation of cDNA-resistant cell poly(A)-containing RNA hybrids. Approximately 30 ng of [^{3}H]cDNA, prepared as described in *Step A*, were hybridized to 165 μg of poly(A)-containing RNA from resistant (AT-3000) cells (approximately 100-fold excess of dihydrofolate reductase-specific RNA sequences) in a 600-μl reaction mixture containing 0.12 M $NaPO_4$, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After incubation at 68° to a R_0t of 0.8, the reaction mix was diluted to 3.7 ml with 0.12 M $NaPO_4$ plus 25 μg each of native and denatured salmon sperm DNA. At this point, 43% of the cDNA was resistant to S1 nuclease. Single- and double-stranded material was fractionated by hydroxylapatite chromatography as described above, and approximately 35% of the radioactivity was recovered in the 0.5 M $NaPO_4$ (double-stranded) fraction. RNA was removed from this fraction by alkaline hydrolysis as described under "Experimental Procedures." Following neutralization, 25 μg of *E. coli* tRNA carrier was added and $NaPO_4$ was removed by chromatography on Sephadex G-100. The void volume was pooled, concentrated by ethanol precipitation, and dissolved in H_2O.

As a final purification step (*Step B*, Fig. 4), the cDNA selected in Step A was hybridized to a 140-fold mass excess of resistant cell poly(A)-containing RNA, to a final R_0t of 0.8 mol-s/liter. Hybridized sequences were then isolated by chromatography on hydroxylapatite. As can be seen in Fig. 3*B*, cDNA complementary to RNA sequences that are highly abundant in resistant cells (putative dihydrofolate reductase-specific sequences) are hybridized at this R_0t and are therefore selected. However, both the low level of cDNA sequences that appear to hybridize to less abundant RNA sequences and the nonhybridizable material selected by the previous step are excluded. Approximately 35 to 40% of the cDNA was recovered in the double-stranded fraction in this step. When analyzed by alkaline, isokinetic sucrose gradient centrifugation (see "Experimental Procedures" for details) this material sedimented as a symmetrical peak at 5.4 S, with a calculated size of approximately 350 bases. The specificity of this purified cDNA fraction was then analyzed as described below.

Specificity of the Purified cDNA—The cDNA selected by the final step of the purification procedure (Step B) should represent the portion of the cDNA resulting from the previous step that had highly accelerated kinetics when hybridized to RNA from resistant cells as opposed to that of sensitive cells. As expected, this material still hybridizes to excess poly(A)-containing RNA from resistant cells at a rate approximately 200-fold greater than to that of sensitive cells (Fig. 5). However, these hybridization reactions now approach 100% with kinetics suggestive of a single, pseudo-first order reaction. This result suggests, but does not prove, that the purified cDNA preparation consists mainly of sequences complementary to a single species of mRNA. (See below for further discussion of this point.) Since the purification procedure would enrich for any cDNA sequence complementary to mRNA present at high abundance in the resistant but not the sensitive cells employed in the procedure, we further defined the specificity of the purified cDNA by analyzing the hybridization of this material to poly(A)-containing RNA

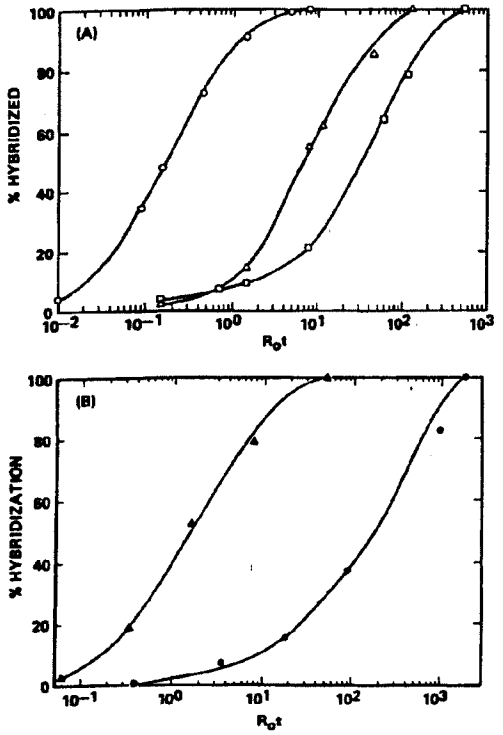

FIG. 5. *A*, hybridization of purified cDNA to RNA from sensitive, resistant, and partially revertant lines of S-180 cells. Poly(A)-containing RNA isolated from S-3 (0.17 μg to 12 μg/sample, □——□), AT-3000 (0.17 μg to 2.2 μg/sample, ○——○), and Rev-400 (0.17 μg to 5 μg/sample, △——△) were reacted with 30 pg (300 cpm) of the purified [^{3}H]cDNA (selected as described in the legend to Fig. 4) and the extent of hybridization at the indicated R_0t values measured by hydrolysis with S1 nuclease. (See "Experimental Procedures" for details.) Endogenous resistance of the purified cDNA to S1 nuclease hydrolysis was approximately 1.5%. *B*, hybridization of purified cDNA to RNA from sensitive and methotrexate-resistant lines of mouse L1210 lymphoma cells. Poly(A)-containing RNA isolated from the L1210S (0.055 μg to 5.5 μg/sample, ●——●), and L1210 RR500 (0.004 μg ot 4 μg/sample, ▲——▲) cell lines were reacted with 40 pg (400 cpm) of purified [^{3}H]cDNA as described in the legend to Fig. 4.

extracted from several other cell types in which dihydrofolate reductase levels vary widely as a function of methotrexate resistance.

By growing resistant sarcoma 180 cells in the absence of methotrexate for 400 cell doublings (12), we have established a partially revertant line (Rev-400) in which the level of dihydrofolate reductase has declined to an apparently stable[3] value approximately 10-fold greater than that of sensitive cells (Table I). This decrease is also accompanied by a decrease in the relative synthesis of dihydrofolate reductase (12) and the level of translatable dihydrofolate reductase mRNA (13). Comparison of the $R_0t_{1/2}$ value for the reaction of the purified cDNA with RNA from partially revertant cells (Fig. 5*a*) to those observed in the reactions with sensitive and resistant cell RNA indicates that in each of these lines the abundance of mRNA sequences complementary to the purified cDNA is proportional to the relative level of dihydrofolate reductase (Table I). In addition, we have also examined hybridization of

[3] R. Kaufman, unpublished observation.

TABLE I

Relative level of dihydrofolate reductase activity, mRNA and gene copies in S-180 and L1210 lines

The origin of each of the lines noted above is described under "Experimental Procedures" and "Results." In each column, values are normalized to those of the sensitive line which was taken as 1. Dihydrofolate reductase-specific activity was assayed as described previously (12). The relative abundance of dihydrofolate reductase mRNA sequences in the S-180 lines was determined from the inverse of $R_0t_{1/2}$ values for the reactions shown in Fig. 5*A*. The relative number of dihydrofolate reductase gene copies in the S-180 and L1210 lines was determined from the inverse of the $C_0t_{1/2}$ values of the reactions shown in Figs. 8 and 9, respectively. In order to estimate the $C_0t_{1/2}$ of the reaction with L1210S DNA (Fig. 9), we assumed this reaction would proceed to the same extent as the others.

Line	Relative dihydrofolate reductase		
	Specific activity	mRNA sequences	Gene copies
S-180			
S-3	1	1	1
AT-3000	250	220	180
Rev-400	10	7	10
L1210			
S	1		1
RR(+mtx)	35		45
RR(−mtx)	35		35

the purified cDNA to excess RNA from both murine L1210 lymphoma cells, as well as a 25,000-fold methotrexate-resistant subline, L1210 RR500 (Fig. 5*b*). Relative to the parental line, the RR500 subline has an approximately 80-fold greater level of dihydrofolate reductase activity that is associated with an increase in both the level of dihydrofolate reductase synthesis and translatable dihydrofolate reductase mRNA (data not shown). The data in Fig. 5*b* indicate that the purified cDNA hybridizes to excess RNA from the L1210 RR500 line at a rate approximately 100-fold greater than that observed with RNA from the sensitive parental line. Therefore, sequences complementary to the purified cDNA are again present at a level proportional to the relative dihydrofolate reductase content of these two cell types. Thus these results, which link the abundance of RNA sequences complementary to the purified cDNA to dihydrofolate reductase levels in a variety of different cell lines, strongly suggest that the purified cDNA preparation consists specifically of sequences complementary to dihydrofolate reductase mRNA. This conclusion is substantiated by two independent lines of evidence which are described below.

We did not size-fractionate either the RNA or the cDNA in the purification procedure. Therefore, another criterion of the specificity of the purified cDNA would be to show that it is specifically complementary to RNA the size of dihydrofolate reductase mRNA. Thus, total RNA from resistant cells was fractionated on isokinetic sucrose gradients, and an equal portion of each fraction was hybridized to purified cDNA under conditions where the per cent hybridization is roughly proportional to the concentration of complementary RNA sequences (30). Other aliquots were used to assay both total and dihydrofolate reductase mRNA activity. As shown by the data in Fig. 6, the purified cDNA hybridizes specifically to a size class of RNA that is distinct from that of total mRNA activity and identical to that of translationally active dihydrofolate reductase mRNA.

Finally, we analyzed the hybridization of the purified cDNA

to both total polysomal RNA from resistant cells, as well as RNA from the same preparation in which dihydrofolate reductase sequences were either enriched or depleted by immunoprecipitation of dihydrofolate reductase-synthesizing polysomes. As shown in Fig. 7, the immunoprecipitation procedure specifically enriched for RNA sequences complementary to the purified cDNA. This result links the specificity of the purified cDNA to the previously demonstrated specificity of the anti-dihydrofolate reductase antibody (12). More importantly, however, the abundance of RNA sequences complementary to the purified probe was directly proportional to the level of dihydrofolate reductase mRNA in each of these fractions (Table II). Therefore, RNA sequences complementary to the purified cDNA are enriched identically to dihydrofolate reductase mRNA by immunoprecipitation of dihydrofolate reductase-synthesizing polysomes.

In summary, in all of the kinetic analyses described above the purified cDNA hybridized with excess RNA to essentially 100% with kinetics suggestive of a single, pseudo-first order reaction. Although this observation suggests that the cDNA is complementary to a single species of mRNA, indistinguishable reaction kinetics would be observed if the cDNA preparation consisted of several different sequences, all of which had complements present at identical abundance in the driver RNA. However, in all RNA preparations that we have examined, the rate at which the purified cDNA hybridized was proportional to the level of dihydrofolate reductase mRNA activity. This was true both in experiments where the abundance of dihydrofolate reductase mRNA varied due to biological factors (*i.e.* in resistant, sensitive, and revertant cells) as well as in experiments where the abundance of these sequences was experimentally manipulated (*i.e.* gradient fractionation or immunoprecipitation). We feel that it is extremely unlikely that any other mRNA would respond identically to translationally active dihydrofolate reductase mRNA with respect to all of these criteria. Therefore, we conclude that our purified cDNA preparation is comprised specifically of sequences complementary to dihydrofolate reductase mRNA.

We have also used the procedure described in Fig. 4 to purify cDNA sequences that were prepared from total poly(A)-containing RNA of resistant (AT-3000) cells that was not further enriched for dihydrofolate reductase mRNA. The cDNA purified in this way was again dihydrofolate reductase-

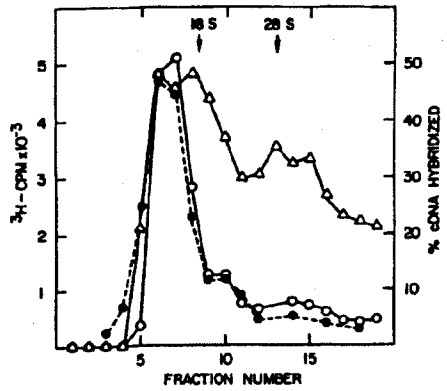

FIG. 6. Hybridization of purified cDNA to size-fractionated resistant cell RNA. Total cytoplasmic RNA from AT-3000 cells (200 μg) was fractionated on isokinetic sucrose gradients as previously described (13). Each fraction was adjusted to contain 0.3 M NaCl and 15 μg of *E. coli* tRNA carrier, and nucleic acids were subsequently precipitated overnight at −20° by the addition of 2 volumes of ethanol. Precipitates were washed twice with 70% ethanol plus 0.1 M NaCl, lyophilized, and dissolved in 100 μl of H_2O. Equal (25 μl) aliquots from each fraction were assayed in the mRNA-dependent reticulocyte lysate system for stimulation of incorporation into total trichloroacetic acid-precipitable material (Δ——Δ) and dihydrofolate reductase (○——○) as described under "Experimental Procedures." Other equal aliquots (3 μl) of each fraction were reacted with 25 pg (250 cpm) of purified [^{3}H]cDNA for 45 min in a final reaction volume of 30 μl. Other reaction conditions and measurement of the extent hybridization in each sample (●- - -●) by S1 nuclease hydrolysis are described under "Experimental Procedures." These conditions were devised so that the maximum extent of hybridization in any sample was less than 50%. Therefore, the per cent hybridization of the [^{3}H]cDNA is roughly proportional to the concentration of complementary RNA sequences in the corresponding gradient fraction (30).

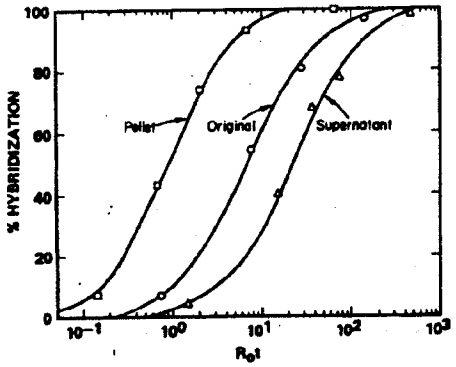

FIG. 7. Hybridization of purified cDNA to RNA extracted from immunoprecipitated dihydrofolate reductase-synthesizing polysomes. Dihydrofolate reductase-synthesizing polysomes were immunoprecipitated from 200 A_{260} units of AT-3000 cell polysomes as described under "Experimental Procedures." Total RNA was extracted from the supernatant and pellet fractions resulting from this procedure, as well as from a reserved sample of the original unfractionated polysomes. RNA from each of these fractions was then reacted with 30 pg (300 cpm) of purified cDNA and the extent of hybridization at the indicated R_0t values determined by hydrolysis with S1 nuclease (see "Experimental Procedures" for details). Hybridization of cDNA to RNA extracted from: pellet (0.115 to 11.5 μg/sample, □——□); supernatant (1.6 to 16 μg/sample, Δ——Δ); original polysomes (2.6 μg to 26 μg/sample, ○——○).

TABLE II

Hybridization to partially purified dihydrofolate reductase mRNA

Samples from each of the fractions described in Fig. 7 were also assayed for stimulation of incorporation into dihydrofolate reductase in the mRNA-dependent rabbit reticulocyte lysate system which was then expressed as a per cent of the total stimulated trichloroacetic acid-precipitable radioactivity as described under "Experimental Procedures." Also shown is the inverse of the $R_0t_{1/2}$ values of each of the corresponding hybridization reactions which is proportional to the abundance of complementary sequences in the RNA sample used to drive the reaction. In order to facilitate comparison of the inverse of $R_0t_{1/2}$ and the per cent dihydrofolate reductase synthesis, the values in each column were normalized to the value for the original fraction which was set equal to 1. Normalized values are shown in parentheses.

Sample	$1/R_0t_{1/2}$	% dihydrofolate reductase synthesis
Original	0.14 (1)	3.8 (1)
Supernatant	0.045 (0.32)	1.2 (0.32)
Pellet	1.1 (7.7)	22.5 (5.9)

specific as judged by the criteria described above. This result confirms our assumption that only the level of dihydrofolate reductase mRNA sequences are greatly increased in resistant cells. Furthermore, this result also indicates that the approximately 200-fold increase in the abundance of dihydrofolate reductase mRNA sequences in resistant cells (Fig. 5*a*) is sufficient to allow purification of dihydrofolate reductase-specific cDNA by this method.

This general approach to specific cDNA purification has been used to purify cDNA sequences complementary to RNA sequences that were absent in mutant cells (31) or viruses (32). Our results indicate that this approach can be extended to situations where it is possible to obtain RNA preparations that have been enriched for specific sequences (for example, by induction or partial purification).

Selective Multiplication of Dihydrofolate Reductase Genes in Resistant Lines

Selective Gene Multiplication in Unstable Lines of Methotrexate-resistant Cells—One possible mechanism consistent with the marked instability of the overproduction of dihydrofolate reductase in methotrexate-resistant lines of Sarcoma 180 cells (12) is selective multiplication of the dihydrofolate reductase structural gene (33). In order to test this possibility, DNA prepared from the nuclei of sensitive, resistant, and revertant cells was denatured and allowed to reanneal in the presence of a trace amount of dihydrofolate reductase specific cDNA. The per cent association at various C_0t values was then determined by fractionation of the double- and single-stranded material on hydroxylapatite. We detected no significant differences in the renaturation of the driver DNA from each of these cell types (Fig. 8, - - -) and, in all of these reactions, association of the dihydrofolate reductase-specific cDNA went to approximately 80 to 85% completion with kinetics characteristic of a unique, second order reaction. Association of the purified cDNA with sensitive cell DNA (Fig. 8, ○——○) occurred over roughly the same C_0t range as observed for renaturation of the unique sequence fraction of the genomic DNA suggesting that dihydrofolate reductase genes are present, on the average, at no more than a few copies per cell in this line. However, the dihydrofolate reductase-specific cDNA associated with DNA from resistant cells (Fig. 8, △——△) at a rate approximately 200-fold greater than that with which it associated with sensitive cell DNA. In addition, similar relative rates were obtained when these reactions were assayed by S1 nuclease hydrolysis (data not shown). Thus, the dihydrofolate reductase structural gene is selectively multiplied approximately 200-fold in resistant cells, a level roughly in proportion to the relative increase in the content of dihydrofolate reductase and dihydrofolate reductase mRNA in this variant line (Table I).

Analysis of the association kinetics of the specific cDNA to DNA from partially revertant cells (Fig. 8, □——□) indicates that the dihydrofolate reductase gene copy number is unstable in resistant cells. Comparison of the kinetics of this reaction to those observed for the reaction of the cDNA to DNA from resistant and sensitive cells (Fig. 8, Table I) demonstrates that the number of dihydrofolate reductase gene copies in the partially revertant line has declined to a value approximately 10-fold greater than that of the sensitive cells. Once again, this value is proportional to the level of dihydrofolate reductase in revertant cells relative to sensitive and resistant cells (Table I).

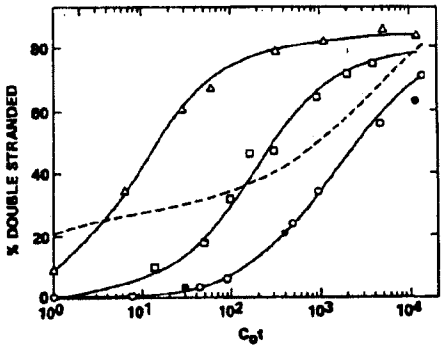

FIG. 8. Association kinetics of purified cDNA with DNA from sensitive, resistant, and partially revertant lines of S-180 cells. DNA was prepared from S-3, AT-3000, and Rev-400 as described under "Experimental Procedures." An aliquot was removed from the S-3 preparation at a point in the preparation immediately preceding NaOH treatment and adjusted to contain 20 μg of poly(A)-containing RNA from resistant cells per mg of DNA. This sample was then processed through the final DNA preparation steps identically to the others. Based on the yield of RNA and DNA from these lines, this ratio of added RNA to DNA approximately represents the relative level of RNA to DNA in these cells. DNA from each of these preparations was melted and allowed to reanneal in the presence of a trace amount of purified [^{3}H]cDNA and the extent of association at various C_0t values measured by chromatography on hydroxylapatite (see "Experimental Procedures" for details). When incubated in the absence of driver DNA, approximately 2% of the [^{3}H]cDNA was retained by hydroxylapatite. Total and double-stranded recoveries of [^{3}H]cDNA from each sample were corrected for this value before calculation of the present double-stranded. The reassociation of the driver DNA from each of these preparations occurred with essentially identical kinetics which are summarized by the *dashed line*. Association of purified [^{3}H]cDNA with DNA from S-3, ○——○; S-3 processed with added poly(A)-containing RNA from AT-3000, ●——●; AT-3000, △——△; and Rev-400, □——□.

In order to show that contamination of the DNA preparations by cellular RNA could not have artifactually led to these results, we demonstrated that the rate with which the dihydrofolate reductase-specific probe associates with sensitive cell DNA was not affected by the addition of a large excess of resistant cell poly(A)-containing RNA to the sensitive cell DNA at a point in the DNA preparation procedure immediately preceding base treatment (Fig. 8, ●——●). Since the amount of added RNA represented considerably more than the maximum possible level of RNA contamination at this point (see legend to Fig. 8), this experiment demonstrates that the base hydrolysis step is sufficient to remove any contaminating RNA.

Selective Gene Multiplication in Stable Lines of Methotrexate-resistant Cells—The 5000-fold methotrexate-resistant L1210RR line of murine L1210 lymphoma cells contains an approximately 35-fold increase in the level of dihydrofolate reductase relative to the sensitive, parental line (Table I). We have also shown that this increase is associated with an increase in the rate of dihydrofolate reductase synthesis and the level of dihydrofolate reductase mRNA activity (data not shown). Increased dihydrofolate reductase levels appear to be a stable property of this resistant line, since we find no significant decrease in this parameter after more than 100 cell doublings in the absence of methotrexate (Table I). The stability of increased dihydrofolate reductase levels observed when these and other lines of methotrexate-resistant cells

were grown in the absence of methotrexate (15) suggested that the alteration leading to increased enzyme synthesis might be a regulatory mutation. In order to test the generality of the selective gene multiplication phenomenon, we quantitated the relative number of dihydrofolate reductase genes in the L1210 lines just described. The data in Fig. 9 indicate that the dihydrofolate reductase-specific cDNA associates with DNA from the L1210RR line grown in the presence of methotrexate (O——O) at a rate approximately 45-fold more rapid than that observed with DNA from the sensitive parental line (Δ——Δ), indicating that the relative number of dihydrofolate reductase genes is approximately 45-fold greater in the resistant line. Again, the relative number of dihydrofolate reductase gene copies is roughly proportional to the relative level of dihydrofolate reductase in these two lines (Table I). We observed only a slight, and probably not significant, decrease (20 to 25%) in the rate with which the probe associated to DNA from the L1210RR line that had been grown in the absence of methotrexate. Therefore, the dihydrofolate reductase gene copy number appears to be relatively stable in this line of methotrexate-resistant cells (Table I).

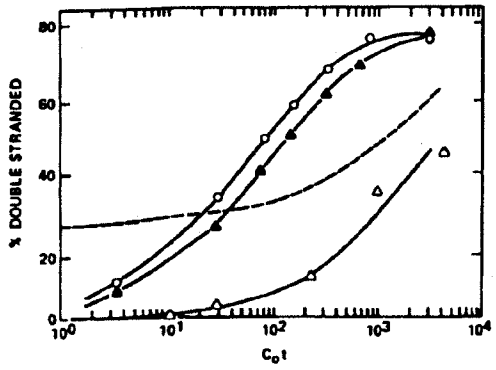

FIG. 9. Association kinetics of purified cDNA with DNA prepared from sensitive and methotrexate-resistant lines of L1210 cells. DNA prepared from various lines of L1210 cells was denatured and allowed to reanneal in the presence of a trace amount of purified [^{3}H]cDNA, and the extent of association at indicated C_0t values determined by chromatography on hydroxylapatite (see "Experimental Procedures" for details). Reassociation of the driver DNA summarized for all three reactions, - - -; association of the purified [^{3}H]cDNA with DNA from L1210S, Δ——Δ; L1210RR, O——O; and L1210RR grown for approximately 100 cell doublings in the absence of methotrexate, ▲——▲.

Thermal Stability of Duplexes between Dihydrofolate Reductase-specific cDNA and DNA from Different Cell Types— The thermal denaturation characteristics of duplexes formed between dihydrofolate reductase-specific cDNA and DNA from either sensitive cells, resistant cells, or mouse liver are essentially indistinguishable (Fig. 10*a*). The T_m values for these reactions range between 81.5° and 82.5°, and in each, the melting profile occurs as a single transition over a relatively narrow temperature range. The T_m of the driver DNA was similar for DNA from S-180 cells (T_m = 84) and human placenta (T_m = 82) (Fig. 10*b*). These reactions also proceeded to the same extent (see legend to Fig. 10). However, although the human DNA contains sequences which can anneal with dihydrofolate reductase-specific cDNA prepared from a murine cell line, the extent of duplex formation (see legend to

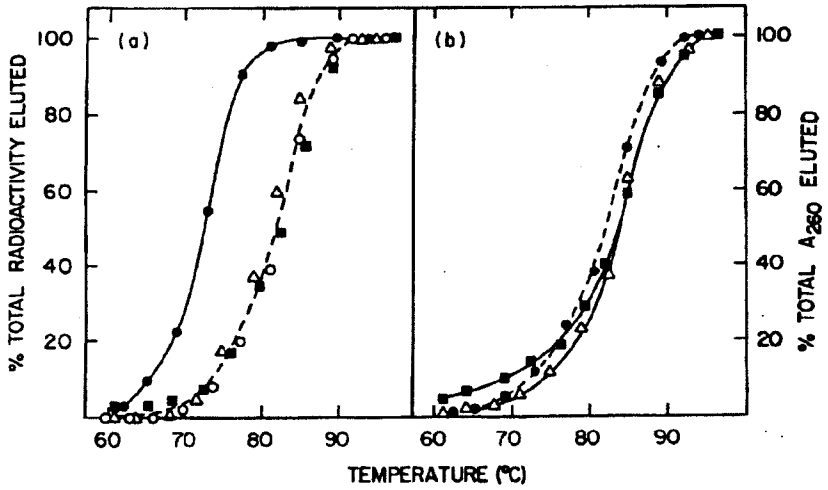

FIG. 10. Thermal denaturation of duplexes formed between dihydrofolate reductase-specific DNA and DNA from various sources. 100 pg of dihydrofolate reductase-specific [^{3}H]cDNA (1000 cpm) was annealed with approximately 1 mg of DNA from AT-3000 (resistant) cells (final C_0t = 1000), S-3 (sensitive) cells (final C_0t = 20,000), mouse liver (final C_0t = 14,000), aborted human placenta (final C_0t = 10,000), and chicken oviduct (final C_0t = 10,000). The final reaction volume was 200 μl, the temperature 68°, and other conditions were as described under "Experimental Procedures." At the indicated C_0t values, reactions were diluted into 5 ml of 0.12 M $NaPO_4$ and adsorbed to 1-g columns of hydroxylapatite which were maintained at 60° with a recirculating water bath. The column was washed with 5 ml of 0.12 M $NaPO_4$ at 60° and subsequently the temperature of the column and wash buffer was raised to 97° in increments of approximately 3°. At each step, the washing procedure was repeated. The resulting fractions were monitored for A_{260} and trichloroacetic acid-precipitable radioactivity as described under "Experimental Procedures." The final percentage of the driver DNA and [^{3}H]cDNA, respectively, that were recovered as double-stranded in each reaction are listed in parentheses below following the appropriate reaction symbols. *Panel a* shows elution of [^{3}H]cDNA and panel *b* show elution of driver DNA from reactions driven with DNA from: AT-3000, O——O (50, 82); S-3, Δ——Δ (77, 60); mouse liver, ■——■ (77, 56); human placenta, ●——● (80, 26), and chicken oviduct (85, 0). Data are presented as the cumulative elution of DNA with increasing temperature.

Fig. 10) and the stability of the duplexes (T_m = 72°) was considerably less than those formed with DNA from murine sources (Fig. 10a). Under the relatively stringent conditions used for these reactions, we observed no duplex formation between dihydrofolate reductase-specific cDNA and chicken oviduct DNA (see legend to Fig. 10).

These results suggest that there is little difference in the nucleotide sequence of individual dihydrofolate reductase genes in resistant cells and, furthermore, that these sequences have diverged little from homologous sequences in sensitive cells or mouse liver (confirming the murine origin of the multiplied dihydrofolate reductase genes in resistant cells). Although there is significant divergence between the nucleotide sequences of the human and murine genes, there appears to be sufficient homology to allow use of the murine probe to analyze methotrexate resistance in human tumors.

DISCUSSION

We have shown that in methotrexate-resistant lines of Sarcoma 180 and L1210 murine lymphoma cells increased synthesis of dihydrofolate reductase is associated with increased copies of the dihydrofolate reductase structural gene. More recently we have also found that increased dihydrofolate reductase synthesis in methotrexate-resistant 3T6 cells is also accompanied by a corresponding increase in the number of dihydrofolate reductase gene copies.[4] These methotrexate-resistant lines represent the first reported examples of mammalian cells in which a structural gene that codes for a protein is selectively multiplied.

In order to understand the processes involved with the selective multiplication of dihydrofolate reductase genes in the resistant lines, we should first examine the role of methotrexate in this phenomenon. This folic acid analogue strongly and specifically inhibits dihydrofolate reductase in a competitive manner (34), and therefore indirectly inhibits the *de novo* synthesis of purines, thymidylate, and glycine (35). Hence, exposure to sufficiently high concentrations of methotrexate kills dividing cells. Resistance to this analogue has been found to result from any of several mechanisms (36), but the most frequently reported for mammalian cells is an increased cellular content of dihydrofolate reductase (2–11). In this case resistant cells accumulate sufficiently high dihydrofolate reductase levels to maintain some free enzyme activity in the presence of the drug (37). Highly resistant lines with greatly increased levels of dihydrofolate reductase (such as those described in this report) have never been selected in a single step. The common method for obtaining such lines involves either gradually increasing the concentration of methotrexate in the medium (3) or progressing in several steps (6), each step using a 10- to 20-fold greater concentration of the drug than is required to inhibit growth by 50%. In the latter case it is possible to estimate the frequency of resistant variants in the cell population. It has been our observation, as well as those of other laboratories (6, 38), that in the initial step this frequency is low (less than 1 in 10^6).

Several lines of evidence suggest that methotrexate does not act directly to induce or maintain the increased synthesis of dihydrofolate reductase in resistant lines. 1. Simple exposure of cells to methotrexate (without selection) has no effect on dihydrofolate reductase synthesis (12). 2. In the methotrexate-resistant lines of L1210 cells that we have studied, as well as a number of other resistant lines (2, 15), increased dihydrofolate reductase synthesis is a stable property and does not decline when cells are grown in the absence of the drug. 3. The kinetics of the decrease in dihydrofolate reductase synthesis observed when unstable lines of resistant cells are grown in the absence of methotrexate do not correspond to dilution of the drug from the cells (12). 4. The decrease in dihydrofolate reductase synthesis observed when unstable lines are grown in the absence of methotrexate is also observed when cells are grown in the continued presence of the drug, but supplemented with a purine source, thymidine, and glycine.[3] Biedler *et al.* (39) have suggested that methotrexate may have mutagenic properties, possibly due to the inhibition of the synthesis of nucleic acid precursors. Such properties could influence the rate (or mechanism) with which resistant variants arise. However, fluctuation analyses done with L1210 cells indicated that in this line methotrexate-resistant variants are generated spontaneously during growth in the absence of the drug (40). In addition, this drug was found to have no mutagenic properties as judged by the *Salmonella* microsome test (41). Therefore, in summary, all available evidence indicates that methotrexate acts only as a selective agent and has no direct role in the resistance (gene multiplication) process.

We propose that exposure of sensitive cells to methotrexate selects for those cells in the population harboring spontaneous multiplications (duplications) of the dihydrofolate reductase structural gene and as a result, increased levels of dihydrofolate reductase. Of course, there are many other conceivable genetic alterations that could lead to increased dihydrofolate reductase levels, including those generating an absolute increase in the transcription rate of the gene or an increased stability of the specific mRNA. However, in all of the lines that we have studied (including 3T6 lines), we observe a proportionality between the relative level of dihydrofolate reductase activity and the relative number of dihydrofolate reductase gene copies (Table I). This result suggests that there is little difference between the activities of individual dihydrofolate reductase genes in sensitive and resistant cells, and that selective gene multiplication is the most important, if not the only mechanism leading to increased dihydrofolate reductase accumulation by these highly resistant lines. A possible explanation for the predominance of this mechanism is that the dihydrofolate reductase gene is expressed at or near the maximum possible activity in sensitive cells, and therefore no type of genetic alteration could greatly increase this activity. Alternatively, the events which lead to duplication or multiplication of dihydrofolate reductase genes may occur at a higher frequency than other types of genetic alterations that would lead to increased expression of a limited number of gene copies.

Clearly, in order to understand the mechanism by which these genes are multiplied, as well as why their number is relatively stable in some lines and not in others, it will be necessary to determine the location and molecular arrangement of the multiple gene copies in the various cell lines. Are they chromosomal or extrachromosomal, and do they exist in tandem arrays or at many locations in the genome? An interesting observation that may reflect on these questions was made by Biedler and her colleagues who consistently detected the appearance of a large homogeneously staining

[4] R. E. Kellems, F. W. Alt, and V. Morhen, unpublished observation.

region associated with specific chromosomes of highly methotrexate-resistant lines of Chinese hamster lung cells (42).[5] In addition, resistance and corresponding high dihydrofolate reductase levels were unstable when these lines were grown in the absence of methotrexate; and, significantly, the size of the chromosomal alteration decreased in parallel to the decrease in enzyme activity (14). It is tempting to speculate that such a chromosomal alteration might correspond to a tandem array of dihydrofolate reductase genes. In some of their resistant lines the specifically altered region represented as much as 6% of the chromosomal complement (14), considerably more than would be necessary to account for an increase in dihydrofolate reductase gene copy number corresponding to the increased enzyme content of the line (approximately 200-fold). However, in bacteria, selected duplication of a specific gene can extend far beyond the vicinity of that gene and involve as much as 20% of the bacterial chromosome (43). If genes other than those coding for dihydrofolate reductase are multiplied in the resistant Sarcoma 180 lines that we have studied, they apparently are not expressed. As judged by both comparison of proteins synthesized by sensitive and resistant cells (12), as well as by the specificity of the cDNA purification procedure (see above), the large increase in dihydrofolate reductase synthesis appears to be unique.

De novo duplication of specific genes in bacteria and phages occurs with relatively high frequencies (43–47); and in these cases duplications appear to be in tandem. A well known example of tandem duplications in eukaryotic cells occurs as a result of unequal crossing over at the bar locus in *Drosophila* (48, 49). If the initial event selected in methotrexate resistance were a tandem duplication of the dihydrofolate reductase gene or alternatively if the genes already existed in multiple, tandem copies in sensitive cells, expansion of the tandem array might occur by homologous but unequal crossover events between dihydrofolate reductase genes on homologous chromosomes (50). However, a more likely mechanism would involve unequal exchanges between sister chromatids. Sister chromatid exchange has been demonstrated to occur in a variety of organisms (51–53) and in *Drosophila* unequal sister chromatid exchanges presumably lead to changes in the number of tandem repeats at the bar locus (54) as well as the number of ribosomal genes at the bobbed locus (55). This process has been discussed in detail as a mechanism for the evolution of repeated DNA sequences (56), the coincidental evolution of members of multi-gene families (57, 58), and the magnification-reduction of the ribosomal gene copy number in *Drosophila* (58, 59). One attractive feature of such a mechanism for the selective multiplication of dihydrofolate reductase genes in methotrexate-resistant cells is that it would be consistent with the multi-step selection procedure necessary to generate these lines.

Alternatively, selective multiplication of dihydrofolate reductase genes may occur by a mechanism which at least initially generates extrachromosomal copies of the multiplied genes. The classic example of such a process in eukaryotic cells is the amplification of ribosomal genes in amphibian oocytes (60). In this case, amplification is specifically regulated as part of a developmental sequence and occurs extrachromosomally, apparently by a rolling circle replication mechanism (61). Other possible amplification mechanisms include reverse transcription of the specific mRNA (62, 63) or disproportionate replication of specific genes (64). The former mechanism may be involved in the production of extrachromosomal copies of mouse mammary tumor virus genes (65) while the latter has recently been implicated in the production of large numbers of extrachromosomal copies of SV40 DNA from the integrated viral genome.[6] One common feature of these mechanisms is that large increases in the number of specific genes might be obtained in a single selective step. In this regard, it will be interesting to measure the absolute number of dihydrofolate reductase gene copies in sensitive cells, and the maximum increase in that number obtainable in a single step.

A selective increase in the number of dihydrofolate reductase genes in resistant cells might also be achieved by the retention of specific chromosomal fragments. Although there are apparently no specific differences between the karyotypes of sensitive and resistant lines (38),[7] chromosome transfer experiments indicate that chromosomal fragments retained by host cells are frequently so small that they may be cytologically undetectable (66, 67). Similarly to dihydrofolate reductase genes in unstable lines, such transferred genetic elements are usually lost rapidly from host cells (1 to 10% loss per generation) (68–71), but can be maintained indefinitely by growth under appropriate selective conditions (70). In addition, prolonged growth of host cells under selective conditions leads to the emergence of lines which stably express the transferred characteristic (66, 70).

Finally, by analogy to bacterial systems, duplication or subsequent multiplication of specific genes (by many of the mechanisms considered above) may also be promoted by flanking sequences (*e.g.* translocatable elements or viral sequences) (72, 73). Such sequences (insertion elements) may be involved in the accumulation of R-factors containing multiple r-determinant segments in chloramphenicol-resistant lines of *Proteus mirabilis*. This phenomenon also shares many features with methotrexate-resistance in Sarcoma 180 cells. High levels of chloramphenicol resistance are unstable in the absence of selection and result from increased production of chloramphenicol transacetylase in association with the selective multiplication of the r-determinant carrying the gene for this enzyme (74, 75).

An intriguing question is why the multiple gene copies are stable in some lines and unstable in others. One possibility is that the multiplication process occurs by a different mechanism in these lines, but recent results indicate that this need not be the case. After growth in the presence of methotrexate for an additional 2 years, the highly unstable lines of methotrexate-resistant S-180 cells described previously (12) appear to have become much more stable.[5] This phenomenon can be explained as follows: whatever the mechanism of gene loss (see discussion below), unstable lines of resistant cells are presumably constantly generating cells with decreased numbers of dihydrofolate reductase genes. Growth of such lines in the presence of methotrexate would maintain a certain average level of dihydrofolate reductase gene copies per cell by eliminating those cells in which the gene copy number (and correspondingly dihydrofolate reductase levels) had decreased below that necessary for survival. Under these conditions,

[5] A similar chromosomal alteration was recently observed in methotrexate-resistant Chinese hamster ovary cells. L. Chasin, personal communication.

[6] M. Botchan, personal communication.

[7] J. Nunberg and R. Kaufman, unpublished observation.

cells in which the gene copy number had become more stable would have an obvious selective advantage (more of their progeny would survive) and eventually outgrow the population.

Loss of chromosomal genes might be associated with specific chromosomal deletions or fragmentations (70). In addition, if the multiple gene copies exist in clusters of tandem repeats in resistant lines, instability in their numbers could be due to the same general types of processes which were considered above for their multiplication. Thus, unequal crossover events would generate as reciprocal products both a cell with increased numbers of dihydrofolate reductase gene copies and one in which the number was reduced; a decrease in the average number of dihydrofolate reductase gene copies per cell would result if in the absence of methotrexate, cells which devoted less of their energy to the production of unnecessarily high levels of dihydrofolate reductase had a selective growth advantage. Tandem duplications in bacterial cells are usually quite unstable (43, 44, 46), presumably due to crossover events between repeats on the same chromosome. Loss of dihydrofolate reductase genes might occur by a similar process, and by analogy to bacterial systems in which such repeats are much more stable in Rec A$^-$ lines (43, 44), stabilization could result from the loss of an enzymatic function that was involved in their excision or exchange. Stabilization might also occur by the inactivation of flanking sequences (by excision or mutation) involved in the multiplication process or by translocation of clustered genes to multiple sites in the genome. Extrachromosomal genes might be lost by a number of different mechanisms. Unstable genetic elements resulting from chromosome transfer experiments (see above) are thought to be extrachromosomal, and recent evidence suggests that stability results from integration of the transferred fragment into the genome of the host cell (67). Stability of extrachromosomal genes, whatever their origin, might be achieved through such a mechanism.

All of our studies were done with murine cell lines; therefore in order to assess the generality of the selective gene multiplication phenomenon it will be necessary to know the mechanism of increased dihydrofolate reductase accumulation in cell lines derived from other organisms (5, 15). The selection of cell lines resistant to highly specific inhibitors of other key enzymes should allow extension of this approach to many different genes. For example, Kempe *et al.* have shown that in certain hamster cell lines, resistance to a specific inhibitor of aspartate transcarbamylase is associated with increased cellular content of that enzyme (76). More recently, this group has found that resistant lines synthesize the enzyme at a greater rate and contain increased levels of the specific mRNA.[8] It will by interesting to know if these lines also contain increased numbers of aspartate transcarbamylase gene copies.

We do not know if the processes leading to selective multiplication of dihydrofolate reductase genes in the permanent cell lines that we have studied have a role in normal cells. Certainly, a mechanism for generating spontaneous and random duplications of genetic material might be important for evolutionary flexibility (77), as well as for the generation of multigene families (33). The unstable lines of resistant Sarcoma 180 cells may, in fact, provide a good model system for studying the evolution and maintenance of multi-gene families; since under appropriate growth conditions it is possible to select lines in which the dihydrofolate reductase gene copy number is increased, decreased, or fixed. In systems where it has been studied, selective gene multiplication as a mechanism for the synthesis of large amounts of differentiated cell proteins has not been observed (78–80). However, other lines of evidence suggest that various types of genomic alterations including duplications, deletions, and translocations may underlie a number of controls of differentiation (see Ref. 81 for further discussion of this point). Our results add further evidence to support the concept that the genome of higher organisms is not constant, but can undergo a variety of changes.

[8] R. Padgett, G. Wahl, and G. Stark, personal communication.

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